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Original article

CDCA7 promotes lung adenocarcinoma proliferation via regulating the cell cycle

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ABSTRACT

CDCA7 is overexpressed in several malignant cancers and is predicted by bioinformatics to be a candidate oncogene in lung adenocarcinoma (LUAD). However, the clinical and biological function of CDCA7 in LUAD has never been investigated. In this study, we used quantitative real-time RT-PCR and immunohistochemistry to determine the expression level and clinical significance of CDCA7. As a result, CDCA7 was significantly overexpressed in LUAD compared to adjacent normal tissues. Furthermore, overexpression of CDCA7 was positively associated with more advanced clinical features. Silencing CDCA7 inhibited cell proliferation in LUAD through G1 phase arrest and induction of apoptosis. In conclusion, CDCA7 can be used as a potential therapeutic target for new biomarkers and LUAD.

1. Introduction

Lung cancer is the most common type of malignant cancer as well as the leading cause of cancer death [1]. The incidence of lung cancer has been continuously increasing during recent decades. Non-small cell lung cancer (NSCLC), which comprises 85% of lung cancer, is the main pathological type and includes two different subtypes, lung adenocarcinoma (LUAD) and lung squamous cancer. Although most LUAD patients have received standard therapies to date, < 15% of LUAD patients survive after 5 years [2,3]. Thus, further investigation on the mechanisms and molecular function of oncogenes is urgently needed to help identify new therapeutic targets.

The CDCA7 gene (Cell division cycle-associated protein 7), also known as JPO1, is located on chromosome 2q31 and encodes a nuclear protein consisting of 371 amino acids [4] in 1997, Dang et al. discovered a new differentially expressed gene, JPO1, in fibroblasts transfected with the Myc gene, using representational difference analysis [5]. The JPO1 gene is periodically expressed in the cell cycle and reaches the highest level between G1 and S and was officially renamed CDCA7 by the Human Genome Nomenclature Committee [6]. Previous studies on CDCA7 genes have focused on the interaction between CDCA7 and Myc [4,7,8]. In 2018, CDCA7 was reported to be a critical mediator of lymphomagenesis and overexpression of CDCA7 predicted poor prognosis in triple negative breast cancer [9,10].

CDCA7 is a recently identified target of myc-dependent transcriptional regulation and is a proto-oncogene that regulates the expression of hundreds of genes involved in cell cycle progression, adhesion, metabolism, and apoptosis. In recent years, several authors, [5,6] have reported high expression of CDCA7 gene in various human malignancies, suggesting that CDCA7 is closely related to various malignant tumors. However, the role of CDCA7 in lung adenocarcinoma has not been studied. Therefore, in-depth study may provide a new target for the treatment of LUAD.

2. Methods and materials

2.1. Date sources and bioinformatics

TCGA (The cancer genome atlas) was jointly launched by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) in 2006. Clinical data, genomic variation, mRNA expression, miRNA expression, methylation, etc. of various human cancers (including tumors including subtypes) are important data sources for cancer researchers.

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The Cancer Genome Atlas (TCGA) data set naming TCGA_LUAD_exp_HiSeqV2-2015-02-24 was downloaded from the website of the UCSC Cancer Browser (https://genomecancer.ucsc.edu/), which contained the data of 511 LUAD patients including 57 paired tissues [11–13]. All standardized mRNA expression values were obtained from the genomic Matrix file. The age, sex, survival, TNM stage, and other clinical data were obtained from the clinical_data file. Differences in CDCA7 expression between LUAD and normal tissues were studied using an unpaired Student's *t*-test. A list of 179 genes with the highest expression correlation with CDCA7 (Pearson's r value $\geq$ 0.5;) was submitted to DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) for KEGG enrichment analysis [14,15]. The probe 224428_s_at(CDCA7) was used for overall survival analysis on kmplot.com (http://kmplot.com/analysis/index.php?p=service&cancer=lung) and we used Median as the cutoff to obtain results.

2.2. Cell lines, cell culture and siRNA transfection

The human LUAD cell lines A549, H1299, PC9, SPC-A-1 and the normal human bronchial epithelial cell line HBE were purchased from Shanghai Life Sciences Research Institute (Shanghai, China). The cells were cultured in RPMI 1640 Medium (KeyGene, Nanjing China) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (KeyGene, Nanjing, China). All the cells were incubated at 37 °C with 5% CO_2. H1299 and PC9 cells were seeded in 6-well plates 24 h before transfection. When confluency reached 60%–70%, cells were transfected with siRNA targeting a specific gene or a negative control (RealGene, Nanjing, China) using Lipofectamine RNAimax reagent (Invitrogen, Carlsbad, CA, USA). Nonsense RNAi was used as a negative control for CDCA7 siRNA. Transfection efficiency was assessed by quantitative real-time RT-PCR and western blotting. Two separate siRNAs were designed, and the sequences were as follows: siRNA-1 of CDCA7: sense 5′- GCCAGATGTCACTAACGAACT-3′, antisense 5′- AGT TCGTTAGTGACATCTGGC-3′; siRNA-2 for CDCA7: sense 5′−CCTCTG ATGACAGTTGTGACA-3′, anti-sense 5′- TGTCACAACTGTCATCAG AGG-3′. And the following nonsense siRNA was used as a control: sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′-ACGUGACACGUUC GGAGAATT -3′.

2.3. Tissue collection and immunohistochemistry

We collected primary NSCLC and adjacent normal tissues from a series of 53 patients who underwent NSCLC surgery in the Department of Jinling Hospital Affiliated to Southern Medical University between 2016 and 2018. No patients received radiotherapy or chemotherapy before surgical resection. The histopathological classification of the tissues was performed by two pathologists in a double-blind manner. All tumor and adjacent normal specimens were rapidly frozen immediately after excision and stored in liquid nitrogen until total RNA and immunohistochemistry were extracted. All tumors and matched normal tissues were validated by experienced pathologists. Clinical characteristics were also collected for each patient. The immunohistochemical staining scores of tissue microarrays were observed by two independent pathologists and scoring was dependent on the intensity and percentage of positive cells. The staining intensity was assessed at 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). This study was approved by the Ethics Committee of the Jinling Hospital Affiliated to Southern Medical University.

2.4. RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from tissues or cells with TRIzol reagent (Invitrogen). About 1.0 μg total RNA was reverse transcribed in a final volume of 20 μl cDNA using the PrimeScript RT Master Mix (Takara). For real-time RT-PCR, SYBR Select Master Mix (KeyGEN) was used, and the reaction was performed in a QuantStudio 6 Flex Real-Time PCR System as follows: initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 92 °C for 15 s and 60 °C for 1 min. Primers of CDCA7 and other associated genes are shown in Table 1 and the housekeeping gene ACTIN was used as a control. Changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1
Primer sequences.

Gene	Sense	Anti-sense
CDCA7	GGGTGGCGATGAAGTTTCCA	GGGGATGTCTTCCACGGAAC
CCND1	CCCGCACGATTTCATTGAAC	AGGGCGGATTGGAAATGAAC
CCNE1	CGGTATATGGCGACACAAGA	AGGGGACTTAAACGCCACTT
CCNE2	CAGGTTTGGAGTGGGACAGT	CTCCATTGCACACTGGTGAC
P21	GCAGACCAGCATGACAGATTT	GGATTAGGGCTTCCTCTTGGA
P27	TGGAGAAGCACTGCAGAGAC	GCGTGTCCTCAGAGTTAGCC
β-ACTIN	GAAATCGTGCGTGACATTAA	AAGGAAGGCTGGAAGAGTG

2.5. Protein extraction and western blotting

Cells were harvested and treated on ice with RIPA lysis buffer (KeyGene, Nanjing, China). Protein concentration was measured using the BCA Protein Assay Kit (KeyGene). Equal amounts of each protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked in 2% bovine serum albumin in the Tris-buffered saline with Tween 20 (TBS-T) for 1 h, then incubated with antibody overnight (4 °C) against CDCA7 (HPA005565-100UL 1:500; Sigma-Aldrich, St. Louis, MO, USA), cyclin D1 (2978, 1:1000; Cell Signaling Technology, Danvers, MA, USA), cyclin E1 (ab7959, 1:200; Abcam, Cambridge, MA, USA), cyclin E2 (11935-1-ap, 1:500; Protein Technologies, Manchester, UK), p21 (SC-397, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) p27 (SC-528, 1:200; Santa Cruz Biotechnology), or GAPDH (D16H11, 1:1000; Cell Signaling Technology). After washing three times with TBS-T 3, the membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Abcam) or goat anti-mouse HRP-conjugated secondary antibody (1:10,000; Abcam) for 2 h at room temperature until the blots were detected by enhanced chemiluminescence (Thermo Fisher Scientific) All experiments were repeated at least three times independently.

2.6. Cell proliferation assay

After 24 h of transfection, the cells were seeded in 96-well plates at 4×10^4 cells per well at a concentration of 100 μL. Then, 20 μL of CCK-8 reagent was added to each well, and the cells were cultured at 37 °C for 2 h. The OD value was then measured at 450 nm on days 1–5. The OD value measured after inoculation of the cells for 6 h on day 1 was taken as a baseline value. For the EdU proliferation assay, we used the kit from Ruibo (Guangzhou, China). After 48 h of cell transfection, the cells were resuspended, counted and seeded in 96-well plates. The percentage of EdU signal in each well of the si-CDCA7 and control groups was measured according to the experimental description procedure. All experiments were repeated at least three times independently.

2.7. Colony-formation assay

Two mL of 200 transfected cells were placed in a fresh 6-well plate and kept in complete medium containing 10% FBS. The medium was replaced every 3 or 4 days. Two weeks later, the cells were fixed with 4% paraformaldehyde and stained with 0.1% Crystal Violet. Then the visible colonies were counted. For each treatment group, each well was evaluated in triplicate.

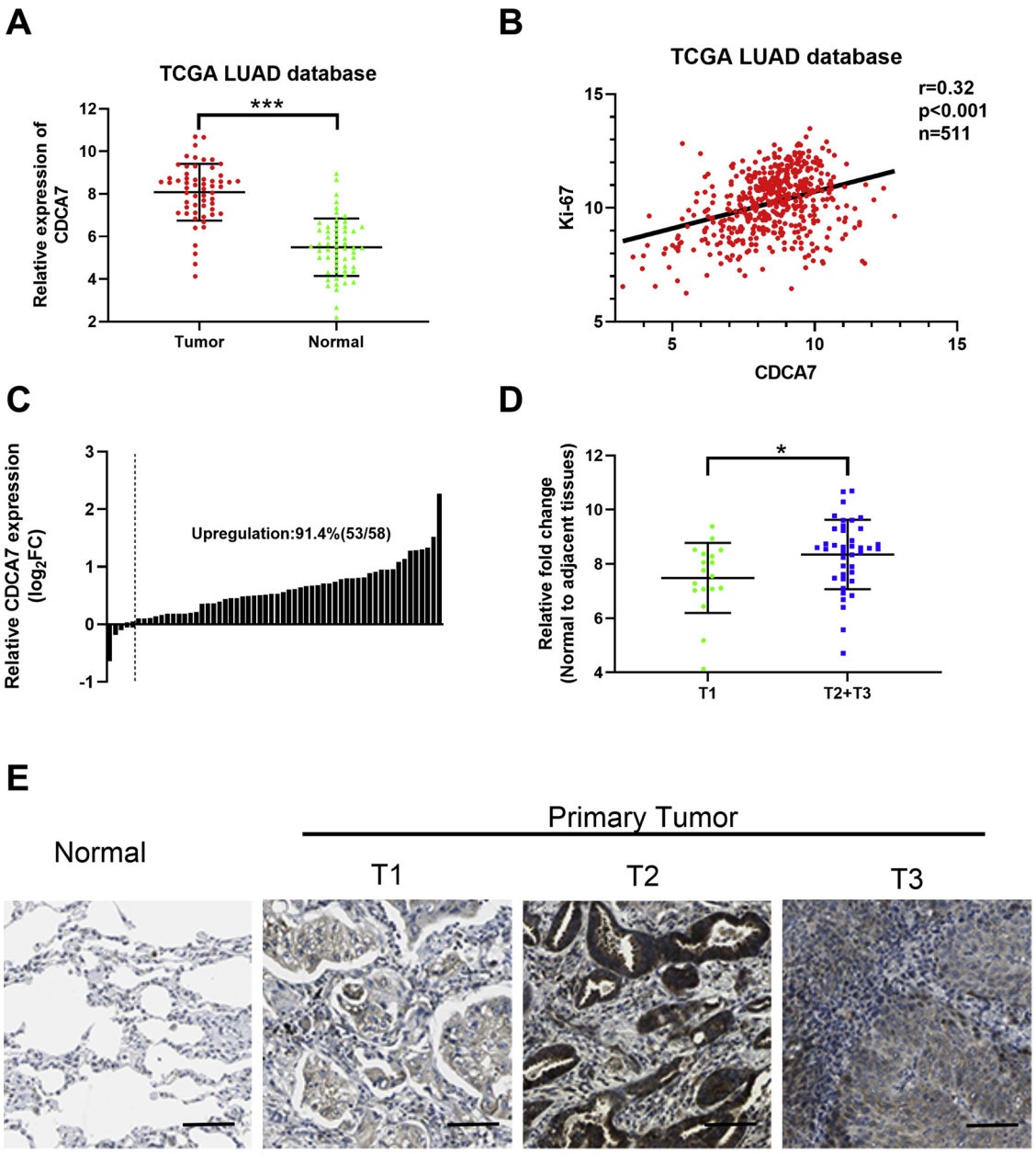

Fig. 1. CDCA7 was overexpressed in LUAD cell lines and tissues.
A. CDCA7 expression was significantly elevated in TCGA LUAD tissues relative to paraneoplastic tissues. **B.** Expression of CDCA7 in TCGA LUAD tissues was highly correlated with the proliferative signaling protein ki67 ($r = 0.32$, $P < 0.001$). **C.** Real-time RT-PCR showed that CDCA7 was highly expressed in 53 of 58 lung cancer tissues. **D.** Expression of CDCA7 was significantly associated with T stage. **E.** Immunohistochemistry showed that CDCA7 expression was significantly elevated in LUAD tissues and was significantly associated with T stage.

2.8. Flow cytometry analysis

Cells collected after transfection with si-CDCA7 or NC after 48 h were fixed with 70% alcohol and stored overnight at −20 °C. The cells were stained with propidium oxide using the Cycle TEST PLUS DNA kit (BD Biosciences) and analyzed by FACScan. The percentages of cells in G1, S and G2/M phases were counted and compared. Apoptosis was measured by an Annexin-V kit. Cells were digested with trypsin, pelleted and washed once with cold PBS. Cells were resuspended in 300 μL binding buffer (1×10^6–5×10^6 cells per condition), then 5 μL Annexin-V–FITC solution was added to the binding buffer. After incubating in the dark for 15 min, nuclei were stained with 5 μL PI for 5 min at room temperature. Finally, 200 μL binding buffer was added, and apoptosis was evaluated by flow cytometry.

2.9. Statistics analysis

SPSS version 20.0 and Graphpad version 7 were used for data analysis, and the results were expressed as mean ± SD. Paired *t*-test was used for paired tissue samples, Student's *t*-test was used for two groups, and one-way ANOVA was used for multiple samples. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Bioinformatics analysis suggests that CDCA7 may be an oncogene in lung adenocarcinoma

We first compared the expression levels of CDCA7 in 57 pairs of lung cancer and paraneoplastic tissues in the TCGA LUAD database. As

Table 2
Correlation of CDCA7 protein expression with clinic pathological characteristics of 53 LUAD cases.

Variables	cases	CDCA7 protein expression		*p*-value
		Low (n = 26)	High (n = 27)	
Age				0. 477
< 65 years	23	10	13	
≥65 years	30	16	14	
Gender				0.695
Male	21	11	10	
Female	32	15	17	
T stage				0.031
T1-T2	36	14	22	
T3-T4	17	12	5	
Lymph node metastasis				0.025
N0	32	18	14	
N1	21	8	12	
TNM stage				
IA–IIA	35	18	17	0.631
IIB- IV	18	8	10	

shown in Fig. 1A, expression of CDCA7 in tumors was significantly increased relative to that in paraneoplastic tissue, $P < 0.001$. The correlation between CDCA7 and the proliferative signal gene KI-67 was used for comparison. Expression of CDCA7 was significantly correlated with that of KI-67 in 505 tumor tissues ($r = 0.32$, $P < 0.001$) (Fig. 1B). According to the data of CDCA7 in TCGA, CDCA7 was highly expressed in tumors and was likely to be closely related to the proliferation of LUAD.

CDCA7 is widely upregulated in LUAD cell lines and correlates with more aggressive clinicopathological characteristics.

To determine expression of CDCA7 in the local population, we extracted total RNA from tumors and paraneoplastic tissues from patients with LUAD within 3 years of resection. Besides, as shown in Table 2, the analysis of clinic pathological characteristics indicated that CDCA7 expression was closed related to the T stage. Real-time quantitative RT-PCR showed that CDCA7 expression in tumor tissues was significantly elevated in 53 of 58 patients (Fig. 1C). Clinical data analysis showed that expression of CDCA7 was significantly correlated with T stage (Fig. 1D). Immunohistochemistry (Fig. 1E) also showed that CDCA7 was significantly elevated in LUAD and was associated with T stage.

Knockdown of CDCA7 inhibits LUAD cell line proliferation via inducing G1-phase arrest and apoptosis *in vitro*.

We observed expression of CDCA7 in LUAD cell lines. Compared with normal lung epithelial cell line HBE, CDCA7 was highly expressed in four LUAD cell lines and was expressed highest in PC9 and H1299. Therefore, these two cell lines were selected to knock down expression of CDCA7 (Fig. 2A, B).

To observe the effect of CDCA7 on the function of LUAD cells. We designed two pairs of siRNAs against CDCA7 to silence its expression. Real-time quantitative RT-PCR and immunoblotting experiments showed that expression of CDCA7 was ideally silenced by > 70% (Fig. 2C, D). To observe whether expression of CDCA7 affected survival of lung cancer patients, we used an online database to compare expression of CDCA7 in 1145 lung cancer patients with the prognosis. High expression of CDCA7 was significantly associated with worse prognosis (HR = 1.48, $P < 0.001$) (Fig. 2E).

CCK-8 analysis showed that knockdown of CDCA7 significantly reduced proliferation of H1299 and PC9 cells (Fig. 3A). The results of the EdU experiment showed that the proliferation of cancer cells was significantly reduced after expression of CDCA7 was attenuated (Fig. 3B). si-cdca7-1-transfected cells showed fewer colonies compared to control siRNA transfected cells (Fig. 3C).

Flow cytometry was used to evaluate the effect of CDCA7 on cell cycle changes and apoptosis. Knockout of CDCA7 significantly increased the percentage of G1 phase H1299 and PC9 cells compared to the control group (Fig. 3D), whereas apoptosis in the si-cdca7-1-treated group increased compared to the control group (Fig. 3E).

3.2. CDCA7 exerts its oncogenic function via upregulation of CCNE1 and CCNE2

One hundred and seventy-nine genes with the highest correlation values for CDCA7 in LUAD were selected from the TCGA dataset. We performed GO and KEGG enrichment analysis on these genes (Fig. 4A, B). Most genes were enriched in the cell cycle pathway. Considering that CDCA7 was periodically expressed in the cell cycle and reached the highest level between G1 and S phases, and knockdown of CDCA7 induced G1 arrest, we measured some G1 or G1/S phase regulators in NC and SI-CDCA7-treated H1299 cells using quantitative RT-PCR and western blotting, including expression levels of CCND1, CCNE1, CCNE2, P21 and P27. The mRNA and protein expression levels of CCNE1 and CCNE2 in si-cdca7-1-transfected cells were significantly lower than those of NC-transfected cells, while CCND1, p21 or p27 did not show any significant change (Fig. 4C, D). We performed a correlation analysis of CDCA7 with these two cyclins in the TCGA lung cancer dataset. Pearson's-related trials showed that the expression of cyclin E1 and E2 was positively correlated with CDCA7 (Fig. 4E).

4. Discussion

In our previous study of the TCGA database and analysis, CDCA7 was significantly expressed in LUAD tissues relative to normal tissues adjacent to the tumor. We analyzed the RNA and protein levels of 58 LUAD samples in our hospital. Real-time RT-PCR and immunohistochemistry showed that the RNA and protein levels of CDCA7 gene in LUAD tissues were significantly different from those of adjacent normal tissues. Based on previous studies, we conducted a preliminary study on the function of the CDCA7 gene. Endogenous CDCA7-expressing cell lines were found in a variety of LUAD cell lines, and expression of CDCA7 was silenced in siRNA-mediated LUAD cell lines with high expression of CDCA7. Subsequently, after performing a cell phenotypic test, it was found that knockdown of CDCA7 significantly inhibited cell proliferation and promoted G1 phase arrest and apoptosis of cancer cells.

CCNE1 protein plays a role in regulating the mitosis of eukaryotic cells by activating and forming a complex with cyclin-dependent kinase 2 (CDK2), which promotes cell passage through the G1/S phase restriction point of the cell cycle. Under normal conditions, Cyclin E1 is expressed and degraded in an orderly manner in the cell cycle. However, under abnormal conditions, the disordered expression of Cyclin E1 in the cell cycle can lead to cell cycle deregulation, interfere with cell mitosis, further lead to cell chromosome instability and promote tumorigenesis. Given that CDCA7 is highly correlated with CCNE1 expression and CDCA7 is capable of promoting lung adenocarcinoma proliferation, we hypothesized that CDCA7 may be located upstream of regulatory CCNE1 and regulate its expression. To test this idea, we can use Chromatin immunoprecipitation technology to detect whether CDCA7 can regulate CCNE1 expression as a transcription factor, or use immunofluorescence or CO-IP (protein complex immunoprecipitationtechnology) to detect that CDCA7 protein can directly bind to CCNE1 and regulate its expression.

After the above experimental results, we found that CDCA7 is likely to play an important role as an oncogene in the progression of lung adenocarcinoma. In view of the fact that CDCA7 may be an important target for LUAD, we can make corresponding antibodies based on the protein structure of CDCA7 to reduce its expression in cells. It can also specifically target cancer with high CDCA7 expression through biotoxin or immunotoxin, thus apply to kill the cancer cells. However, there may be a long way to go from the discovery of basic research to the application to the clinic. Our next step is to verify the role of CDCA7 in solid tumors in mice, and we hope to construct a mouse that knocks out

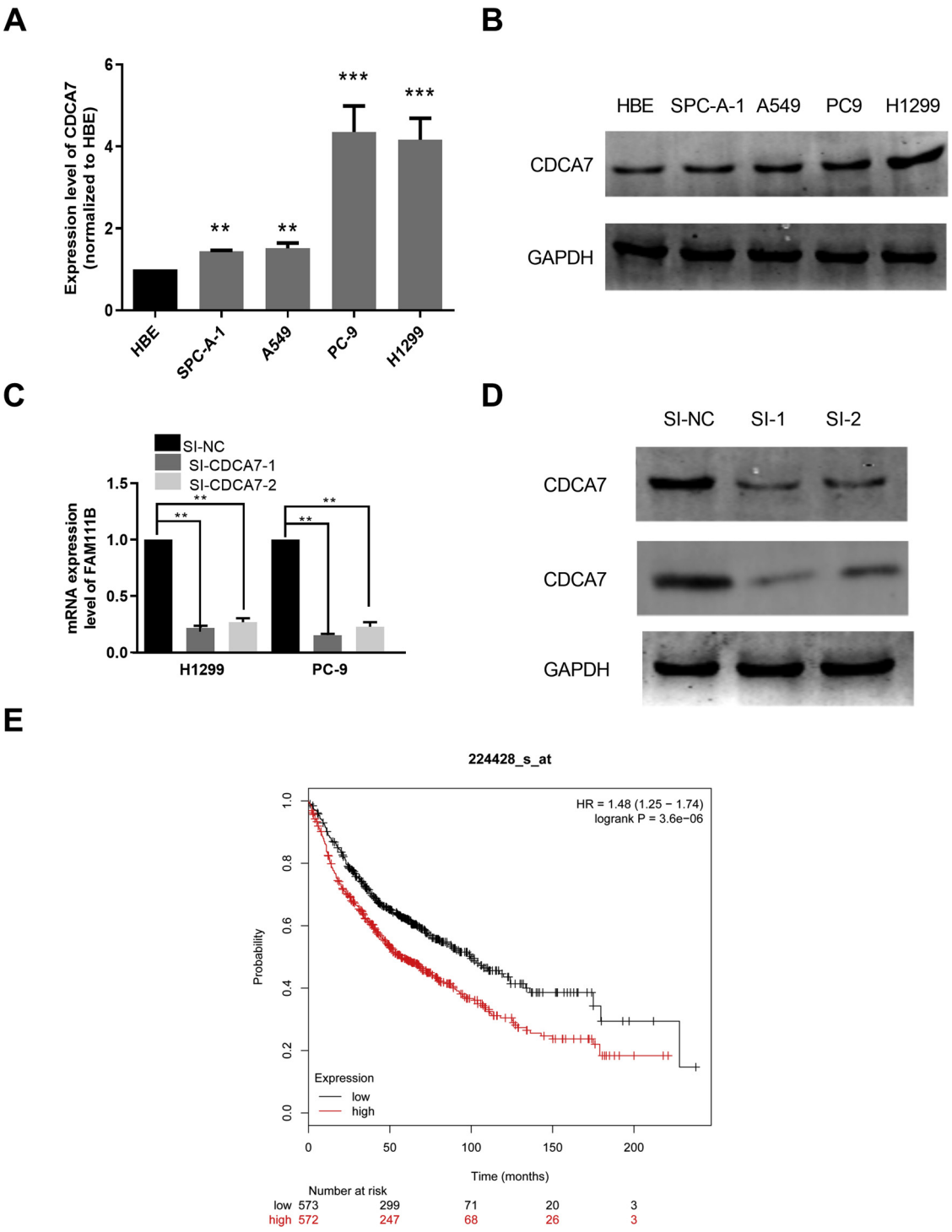

Fig. 2. Expression of CDCA7 in LUAD cell lines and its relationship with overall survival.
A and B. Quantitative real-time RT-PCR and western blotting showed that CDCA7 expression was elevated in LUAD cell lines relative to normal HBE epithelial cells. **C and D.** siRNA successfully silenced CDCA7 expression in H1299 and PC9 cell lines. **E.** Prognosis of patients with high expression of CDCA7 in LUAD was worse (HR = 1.48).

CDCA7. If we can get satisfactory results, we hope to work with biochemists to develop specific oncology drugs targeting CDCA7.

The cell cycle of eukaryotes is regulated by the orderly binding and activation of a series of regulatory factors [16]. The regulation of G1 phase plays a key role in the cell cycle, and c-Myc regulates this key point at multiple levels [17]. As a direct target of Myc, CDCA7 is likely to participate in and promote the regulation of Myc in the cell cycle during tumor development [18]. We therefore measured several G1/S-transition-related genes to explore the potential mechanism. CCND1 and CCNE1 expression was dramatically decreased by knockdown of CDCA7, which was consistent with the result of Pearson's correlation test on CDCA7 with these cyclins.

In conclusion, we showed that the CDCA7 gene may play a tumor-promoting role in LUAD. It is preliminarily speculated that CDCA7 can promote the development of LUAD and even other cancers through cell proliferation and the apoptosis pathway, which provides a new

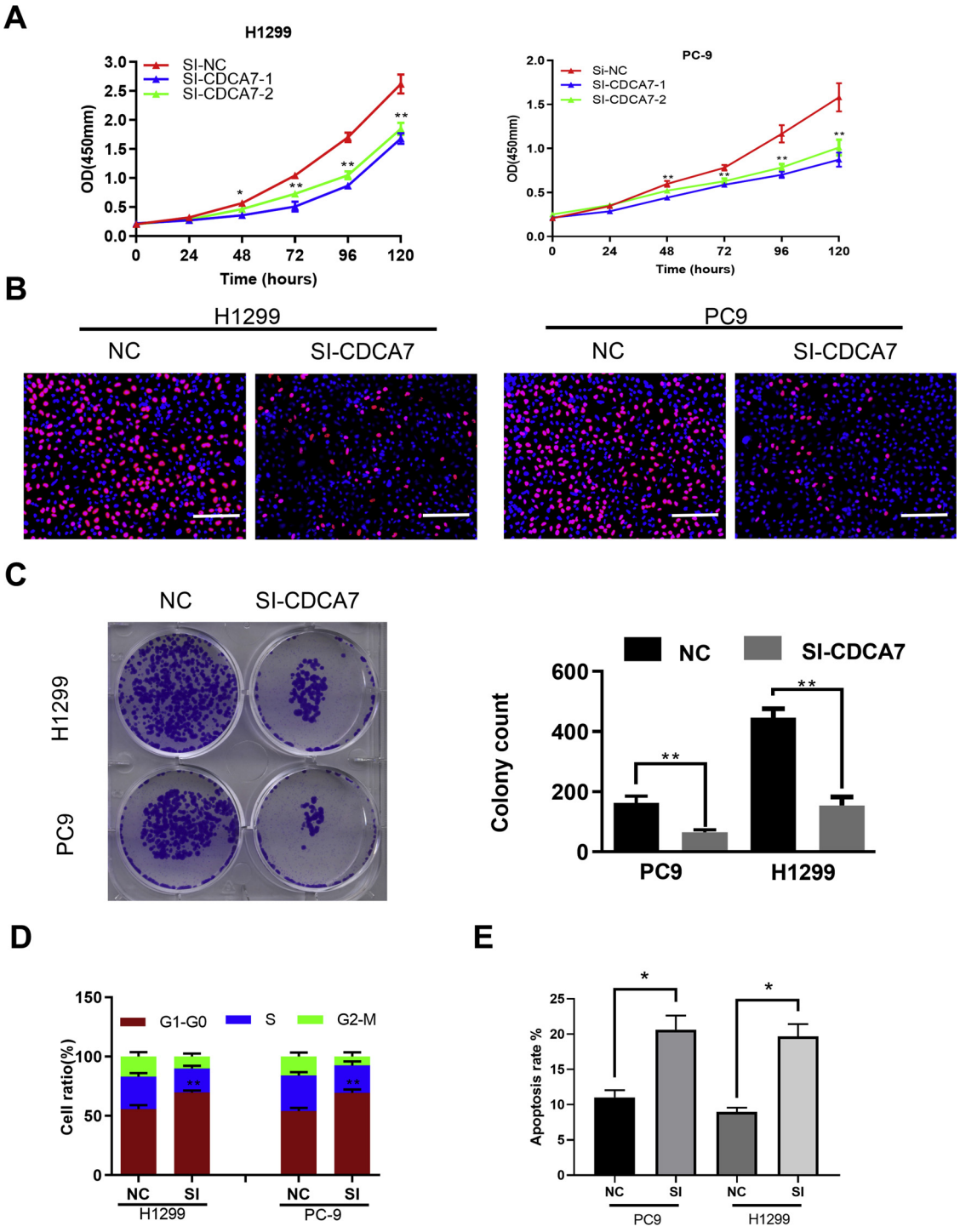

Fig. 3. CDCA7 promoted proliferation of LUAD cells.
A and B. CCK8 and Edu experiments indicated that knockdown of CDCA7 significantly inhibited proliferation of PC9 and H1299 cells. **C.** The number of clones formed after inhibition of CDCA7 expression was significantly reduced. **D.** Silencing CDCA7 expression caused cell cycle arrest in G1 phase. **E.** Inhibition of CDCA7 expression significantly promoted apoptosis.

theoretical basis for further research on the mechanism of CDCA7 gene.

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Declaration of Competing Interest

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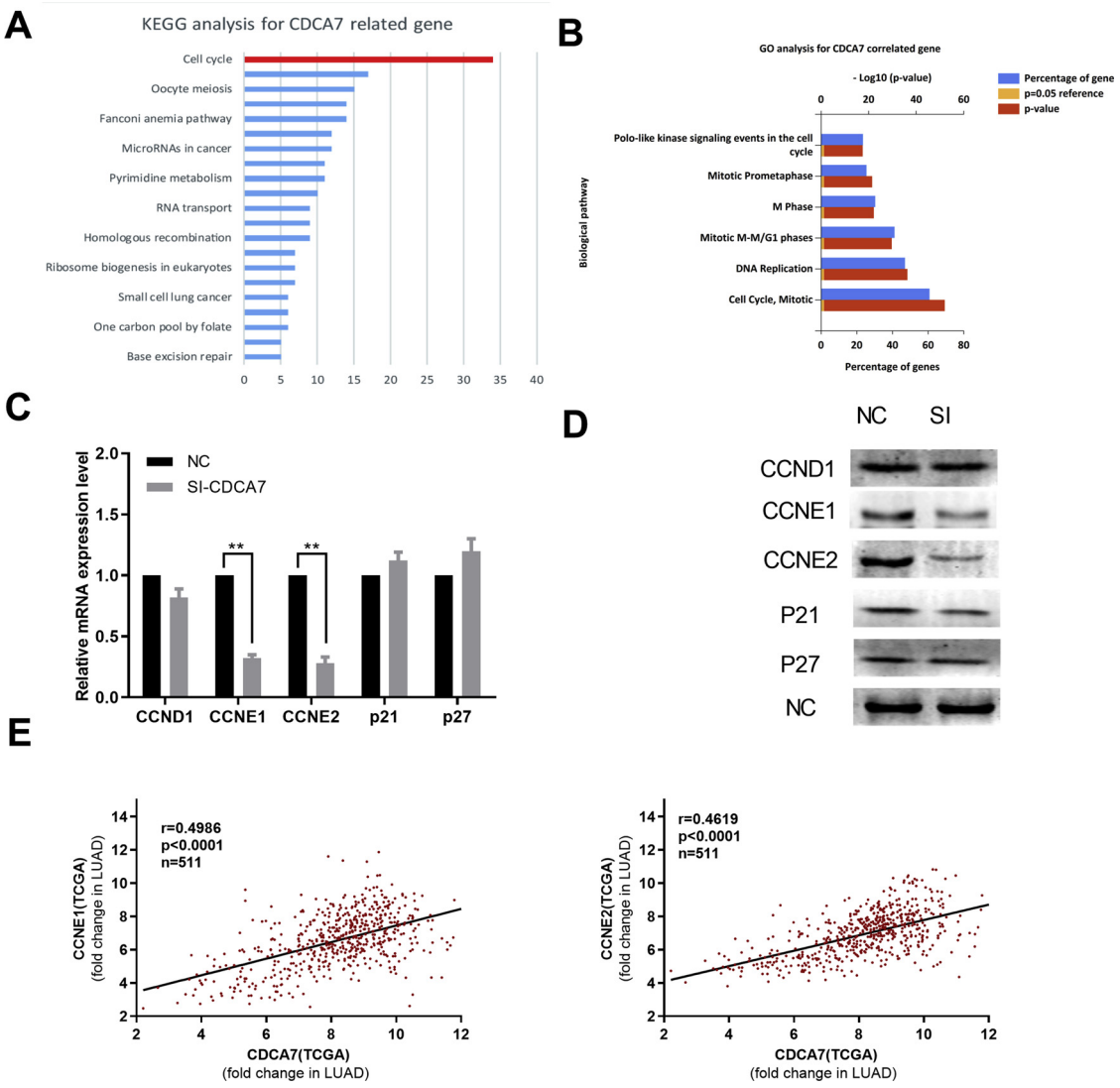

Fig. 4. CDCA7 exerted its oncogenic effect by affecting cyclin E protein.
A and B. KEGG pathway and GO analysis showed that most genes coexpressed with CDCA7 were enriched in the cell-cycle pathway. **C and D.** Quantitative real-time RT-PCR and western blotting showed that as the expression of CDCA7 was inhibited, the expression of CCNE1 and CCNE2 was significantly decreased, while expression of CCND1, p21, and p27 was not obviously changed. **E.** Pearson's test in TCGA database showed that CDCA7 positively correlated with CCNE1 and CCNE2 in LUAD tissues.

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